Detection of extracellular vesicles: size does matter
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CHAPTER 1

Introduction

1.1 Thesis motivation

Cells release small biological sacks filled with fluid, which are called “extracellular vesicles”. The diameter of extracellular vesicles typically ranges from 30 nm to 1 µm\(^{[69,19]}\), the smallest being some 100-fold smaller than the smallest cells in the human body. Fig. 1.1A shows a scanning electron microscopy image of vesicles that are released by an endothelial cell. Because cells release vesicles into their environment, body fluids, such as blood, saliva, and urine, contain numerous cell-derived vesicles. Cells employ vesicles to remove waste and transport and deliver cargo, such as receptors and genetic information, to other cells. Since the cargo allows vesicles to target messages to specific cells, vesicles most likely play a key role in intercellular communication. In addition, the size, concentration, cellular origin, and composition of vesicles in body fluids is changed during diseases. For example, increased levels of vesicles in plasma are associated with thrombosis and metastatic carcinomas\(^{[37,23]}\). The functions of vesicles and their change of properties during disease imply that vesicles have many clinical applications. For example, an abundant concentration of a particular vesicle type could be indicative for the presence of a disease. Such information would enable early recognition of a disease and monitoring the efficacy of therapy. Furthermore, vesicles can be used to deliver drugs specifically to the diseased organ without being cleared by the immune system\(^{[282]}\). For these applications to become reality, we need to gain a profound understanding of extracellular vesicles.

Rose Johnstone appointed her discovery of vesicle formation in 1987 as “Alice in Blunderland”\(^{[74]}\). Although the field of vesicle research is growing exponentially ever since (Fig. 1.1B) and much progress has been made, Johnstone’s statement still applies to the current state of the field. Physical properties of vesicles, such as their size distribution, morphology, refractive index, and concentration are still unknown. For example, the reported concentrations of vesicles in human plasma from healthy individuals differ 100,000,000-fold\(^{[37,314,90,333]}\). However, since normal cell counts differ less than 10-fold between healthy individuals, the true range in vesicle concentrations is probably orders of magnitude smaller.

The main reasons for the differences in the reported concentrations are the small size of extracellular vesicles and the limited sensitivity of detection techniques. Flow cytometry, which is the most widely applied technique to study single vesicles, detects only 1% of all vesicles present. Other techniques measure millions
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Figure 1.1: (A) Scanning electron microscopy image of extracellular vesicles (arrows) that are secreted by an endothelial cell. Image courtesy of Anita Böing. (B) Number of publications per year about extracellular vesicles (bars) fitted by an exponential function (line, $R^2 = 0.98$) [3].

of vesicles simultaneously and are therefore unable to identify sub-populations of vesicles. Over the last decade, novel techniques found their way to the vesicle labs, but their suitability for vesicle detection is not sufficiently tested and understood.

The aim of this thesis is to improve the detection of extracellular vesicles by (1) obtaining insights into fundamental physical properties of vesicles, and (2) gaining a profound understanding of the currently used and novel detection techniques. Since this knowledge is a prerequisite to understand the biological relevance of vesicles in health and disease and increase the clinical usefulness of vesicles, this thesis paves the way to Vesicles’ Wonderland.

1.2 Thesis contents

Chapter 2 contains an introduction to extracellular vesicles. The history, nomenclature, classification, types, properties, functions, and clinical relevance of extracellular vesicles are discussed. In Chapter 3 an overview is provided of 14 currently available and potentially applicable methods for optical and non-optical determination of the size, concentration, morphology, biochemical composition and cellular origin of vesicles. The working principle of all techniques is briefly discussed, as well as their capabilities and limitations based on the underlying physical parameters of the technique. To compare the precision in determining the size of vesicles between the discussed techniques, a mathematical model is developed to calculate the expected size distribution for a reference vesicles population. In Chapter 4 an experimental evaluation is performed of 5 of the 14 methods. The most widely used methods capable of detecting single vesicles are selected, which are transmission electron microscopy, a conventional flow cytometer, a flow cytometer dedicated to detecting sub-micrometer particles, nanoparticle tracking analysis
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and tunable resistive pulse sensing. The accuracy, precision and detection range of these methods are determined. Insight into the capabilities of the applied methods to measure the size distribution of vesicles enabled to explain why the reported concentrations of vesicles in human blood plasma differ 100,000,000-fold.

Chapter 5 further addresses vesicle detection by flow cytometry, which is the most widely used technique to study single vesicles. It is shown that vesicle detection by flow cytometry is partly attributed to swarm detection of smaller vesicles, that is, multiple vesicles are simultaneously illuminated by the laser beam and counted as a single event signal. In addition, the relationship between light scattering and the diameter of vesicles is modeled to demonstrate that the current vesicle gating strategy selects vesicles and cells with a diameter between 800 nm and 2,400 nm.

For optical detection and characterization of vesicles, the refractive index of vesicles is an important but unknown property. The refractive index is important because it relates light scattering to the size and composition of a vesicle. In Chapter 6 a novel method is described to determine both the refractive index and the diameter of single vesicles in suspension.

In chapter 4 it is shown that tunable resistive pulse sensing is the most accurate technique to determine the size and concentration of vesicles in suspension. However, the reproducibility of tunable resistive pulse sensing is not investigated. In Chapter 7 the reproducibility is quantified. Furthermore, a protocol is developed to improve both the reproducibility and sensitivity of tunable resistive pulse sensing.

Because body fluids contain many particles other than vesicles, vesicles require isolation prior to detection. However, isolation of vesicles from particularly plasma is challenging due to the high complexity of this body fluid. In Chapter 8 it is demonstrated that a single step isolation procedure of vesicles from plasma by size-exclusion chromatography can be used. The acquired knowledge on vesicle detection is used to demonstrate that size-exclusion chromatography has excellent recovery and enrichment.

In Chapter 9 the reader is taken beyond the state of the art. The applicability of vesicle detection by specialized techniques, such as Raman microspectroscopy, micro nuclear magnetic resonance, small-angle X-ray scattering and anomalous small-angle X-ray scattering is discussed.

Chapter 10 contains the general discussion, where the major findings of this thesis are placed into the context of the current state of the field. To finish off, the future of extracellular vesicles detection is enlightened.